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A study on the effects of *Alexandriurn minuturn* and *Gymnodiniurn* on the embryonic development of *Sparus macrocephalus*

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Abstract

The toxicity effects of *Alexandriurn minuturn* and *Gymnodiniurn* on the embryonic development of *Sparus macrocephalus* were tested through the toxicological experiments about hatching spawns and developing larvae of *Sparus macrocephalus*. *Alexandriurn minuturn* and *Gymnodiniurn* solution was diluted into three groups (about 3000cell/ml, 1500cell/ml, 500cell/ml). The results showed that the hatch of the spawns was sensitive to both kinds of the algae. And the toxicity results from *Alexandriurn minuturn* were inferior to that from *Gymnodiniurn*. The test of larvae 96-LC50 showed that, larvae was more susceptible to the *Gymnodiniurn* and produced certain resistance to the toxicity of the algae. Both the *Alexandriurn minuturn* and *Gymnodiniurn* restrained body length and weight increase as well as ATPase and GSH-PX enzyme activities of the larvae, while restrain from *Gymnodiniurn* was stronger. In conclusion, the effects on growth and development of *Sparus macrocephalus* spawns and larvae resulted from *Gymnodiniurn* was much higher than that from *Alexandriurn minuturn*.

Keywords: Alexandriurn minuturn; Gymnodiniurn; embryonic development; ATPase; GSH-PX

1. Introduction

Marine culture and fishery resources have been greatly endangered by red tide, especially by toxic red tide which is a direct threat to the living environment and human health [1].The toxins produced respectively by *Alexandrium minitum* and *Gymnodinium sp*., such as PSP and BTX, will do harm to the aquatic organisms and eventually initiate a series of toxic reactions [2, 3]. Physiological function damage and decline may be caused due to the strong response to toxic compounds from external environments in

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the early stage of fish development, when their ability to resist external stresses is weak [4]. The enzyme activity of Na^+-K^+-ATPase which serves as an indicator for animal physiological status has been widely applied in ecotoxicology monitoring [5-7]. Glutathione peroxides (GSH-PX), as one of the important peroxide decomposition enzymes inside, may produce a catalytic disproportionate reaction when suffered interruption from external contamination, which deoxidizes the peroxide into oxide and eliminates oxygen free radicals in cells so as to avoid damages and protect themselves. Researches about the comparison of toxic effects between *Alexandriurn minuturn* and *Gymnodiniurn* are rare [8], although a large number of researchers have released reports on toxic red tidal algae and their toxins at home and abroad [9-11]. The object of this study was to evaluate the effects of *Alexandriurn minuturn* and *Gymnodiniurn* on embryonic development of *Sparus macrocephalus*, in order to compare analysis the toxic effects of these two cyanobactira, and accumulate data about the research on biological effect evaluation of cyanbacteria in the future.

2. Materials and methods

All experiments were carried out at temperature 20.2±2℃, with the pH at 7-8 and the air saturation above 60%. The algae solution at the medium term of the exponent growth period was used for experiments.

2.1. Algae and larvae

Alexandrium minitum (AM-1 Algae plant) was provided by Dr H Chou of Taiwan University. *Gymnodiniurn sp.* was provided by Jinan University. The algae were grown in f/2 medium in flasks, at temperature 20±1℃, 3000lx with 12h-12h light-dark cycle. The larvae of *Sparus macrocephalus* were from Jiangsu fisheries Research Institute fish farms. The newly larval fish was for four days of hatched after hatching the bloodstock direct produced eggs.

2.2. Experimental seawater

Experimental water was the natural seawater got from fish farms, the main salinity was 22. Prior to the experiments, the seawater was also subjected to cotton filtration, boiled for sterilization and air saturation.

2.3. Experimental designs

Each mother alga solution was diluted into three concentrations according to certain proportion. The mixture was divided into 3 cell density groups as 30000 cell/ml, 15000 cell/ml and 7500 cell/ml. The group with seawater only was regarded as the control group. 20 percent of the total liquid was changed every day.

The algae solution as mother liquor was mixed with seawater dilutes according to certain proportion. The mixture was divided into 3 cell density groups as 30000 cell/ml, 15000 cell/ml and 7500 cell/ml. The group with seawater only was regarded as the control group. In each concentration group, there were 3 samples. There were about 100 no open-mouth larvae (which were just hatched out) in 5000ml experimental liquids. 20 percent of the total liquid was changed every day. The larvae's conditions about the growth, movement, outward appearance deformity and death were observed for 96 hours. The larvae were taken out randomly and kept in the liquid nitrogen container every 24 hours.

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seawater was regarded as the control group. In each concentration group, there were 3 samples. There were about 200 open-mouth larvae (which larvae's vittles bag was disappeared, the average body-long was 4.34mm, the average weight was 0.0062g. Lips open degree was 0.3mm) in 5000ml experimental liquids. 20 percent of the total liquid was changed every day. The larvae were fed with rotifer one time every day. The growth, moving, outward appearance deformity and death of the larvae were observed for 7 days. 50 larvae were taken out randomly and kept in the liquid nitrogen container every day. The larvae samples were determined and analyzed the activities of the antioxidant system enzymes GSH-PX and ATPase. 5 larvae were taken out randomly every 24 hours to measure full-length and body weight (wet weight g). Full-length and body weight on growth rate were calculated。

$$\text{Full-length of day on growth rate} = (\lg L_2 - \lg L_1) \times 100/0.434\ (t_2 - t_1) \quad (1)$$

$$\text{Body weight on growth rate} = (\lg W_2 - \lg W_1) \times 100/0.434\ (t_2 - t_1) \quad (2)$$

where t_1, t_2 are regarded as number of days, L_1, L_2 are regarded as full-length at period t_l, t_2, respectively, while W_l, W_2 the full-length at period t_l, t_2 respectively.

The activities of GSH-PX and Na^+-K^+-ATPase were determined by special Reagent box bought from Nanjing Jianchen Bioengineering Research Institute. The activity of GSH-PX was defined as inn 0.1ml tissue protein reaction at 37℃ for 5 minutes, excluding the role of non-enzymatic reaction, the concentration of GSH reduces 1μmol/L in reaction system. It was the amounts for 1 GSH-PX vigour unit. The definition of Na^+-K^+-ATPase activity：When 1mg tissue protein decomposed ATP and produced 1μmol inorganic phosphorus in one hour, the amount of ATP was 1 Na^+-K^+-ATPase vigour unit. SPSS bio-statistical analysis software was used, and one-factor analysis of variance was selected to test the difference between the data obtained from various sampling groups. $P<0.05$ was significant difference.

3. Results

3.1. Impacts on the hatch of spawns

The results of impacts on *Sparus macrocephalus*' spawn hatch in *Alexandriurn minuturn* and *Gymnodiniurn* solutions were shown in Table 1. It revealed that during the hatching process, not only the *Alexandriurn minuturn* but also the *Gymnodiniurn* showed dose-effect relationship: eggs' hatching rate declined with increasing algae concentrations, while the mortality and teratogenicity were just on the contrary. The hatch and mortality rate between the *Alexandriurn minuturn* and *Gymnodiniurn* groups showed few differences ($P>0.05$) at the same concentration, yet the teratogenicity rate showed a notable difference ($P<0.05$).

Table1. Subacute test of Alexandrium minitum and Gymnodinium sp on eggs of Sparus macrocephalus.

Species	Algae concentration (cell/L)	Mortality (%)	Hatchability (%)	Malformation (%)
Alexandrium minitum	7500	14.92±1.04	85.08±7.21	4.49±0.21
	15000	19.27±5.77	80.73±4.96	5.23±0.13
	30000	31.47	68.53	8.29
Gymnodinium sp	7500	16.12	83.88	7.01
	15000	20.43	79.57	9.63
	30000	41.21	58.79	11.01

3.2. Experiments on the fish fry 96H-LC50

Results of the acute toxicity test of *Alexandriurn minuturn* and *Gymnodiniurn* on juvenile black sea bream (*Sparus macrocephalus*) were listed in Table 2, which indicated that *Sparus macrocephalus* was more sensitive to *Gymnodiniurn* than to *Alexandriurn minuturn*. In the same time, the semi-lethal concentration values of the two algae had a discrepancy of 20%, and the discrepancy between them were significant (P<0.05). Also we could find that the toxicity fastness was produced by the larvae of *Sparus macrocephalus* as time extended, and 24 hours had the highest toxic effect concentration, while the 96 hours the lowest.

Table 2. Acute toxicity of Alexandrium minitum and Takayama pulchellum on larvae of Sparus macrocephalus.

Contact time	LC50		95 % CL	
	Alexandrium minitum	*Gymnodinium sp*	*Alexandrium minitum*	*Gymnodinium sp*
24h	21500	17900	18300–38600	15300–31800
48h	19600	13400*	15600–31700	13800–29300
72h	14700	10200*	14100–26100	10700–22600
96h	13900	9400*	13000–24500	8700–20600

* Significance level P<0.05

3.3. Subacute effects to the development of Sparus macrocephalus' larvae

The larvae's full-length and weight had been restrained by *Alexandriurn minuturn* and *Gymnodiniurn*, especially when the concentration of algae reached to 30000 cell/L, the larvae in them had a growth rate of 0.4054 and 0.3714 per day respectively, which were lower than that of the control groups(P<0.05)(Table 3). Also, the length growth rate got to the significant level (P<0.01) in the same conditions. Furthermore, the ability of *Alexandriurn minuturn* in restraint was weaker than that of *Gymnodiniurn* at the same concentration.

Table 3. Effects on the overall length and weight growth test on Alexandrium minitum and Takayama pulchellum on larvae of Sparus macrocephalus.

Algae concentration (cell/L)	*Alexandrium minitum*		*Gymnodinium sp*	
	Length day growth rate (L)	Weight day growth rate(W)	Length day growth rate (L)	Weight day growth rate(W)
0	0.6192	2.0827	0.6192	2.0827
7500	0.5402	1.6333	0.5029	1.6177
15000	0.5012	1.5477	0.4147	1.4544
30000	0.4054*	1.4223**	0.3714*	1.3667**

* significance level P<0.05 * *significance level P<0.01

3.4. The effects to the larvae's Na+-K+-ATPase activity

The ATP enzyme activity in larvae that exposed to the *Alexandrium minitum* was slightly lower than the control groups， with no significant (P>0.05) (Table 4), while the one in *Gymnodinium* was significant (P<0.05). The ATP enzyme activity in larvae was descending as the concentration increasing, which showed a significant dose-effect relationship.

Table 4. Effects of Alexandrium minitum and Gymnodinium sp on the Na+-K+-ATPase activity in larvae of Sparus macrocephalus.

Species	Algae concentration (cell/L)			
	30000	15000	7500	0
Alexandrium minitum	2.180±0.032	2.360±0.032	2.398±0.032	2.513±0.032
Gymnodinium sp	1.702±0.032	1.907±0.032	2.081±0.032	2.513±0.032

Inhibition rate of *Alexandrium minitum* and *Gymnodinium* stress on Na^+-K^+-ATPase was shown in Table 5.It indicated that the inhibition of *Alexandrium minitum* was weaker compared to the control groups, with a rate of 14% below. The toxic effect of *Gymnodinium* was much stronger than *Alexandrium minitum*， with the inhibition rate may reach to 32.29%.

Table 5. Inhibition rate of Alexandrium minitum and Gymnodinium sp stress on Na+-K+-ATPase.

Concentration (cell/L)	*Alexandrium minitum*		*Gymnodinium sp*	
	ATP enzyme activity 95 % CL	Inhibition rate (%)	ATP enzyme activity 95 % CL	Inhibition rate (%)
0	2.5934±0.0832	0. 00	2.5934±0.2269	0. 00
7500	2.4346±0.0367	4.58	2.0289±0.0525*	17.18
15000	2.3877±0.0283	6.10	1.8422±0.0879*	24.12
30000	2.3758±0.1953	13.25	1.5864±0.1152*	32.29

*: notable difference(P<0.05)

3.5. The effects to the larvae's GSH-PX activity

The GSH-PX activity of larval fish under various concentrations of *Alexandrium minitum* and *Gymnodinium* showed a certain degree of decline compared to the control groups (Table 6). Particularly when it was 30000 cell/ml high, the inhibition to GSH-PX activity was significant (P<0.05), while the group 15000 cell/ml and 7500 cell/ml no significant (P>0.05), which indicated that the dose-effect relationship was not remarkable unless it reached a certain threshold. The inhibition from *Gymnodinium* was much stronger than that of *Alexandrium minitu*.

Table 6. Effects of Alexandrium minitum and Gymnodinium sp on the GSH-PX activity in larvae of Sparus macrocephalus.

Species	Algae concentration (cell/L)			
	30000	15000	7500	0
Alexandrium minitum	18.571±0.0032	27.748±0.032	28.357±0.032	31.414±0.032
Gymnodinium sp	16.875±0.032	24.121±0.032	25.289±0.032	31.414±0.032

4. Discussion

The black porgy eggs' membrane layer was a protective barrier against external interference. The results showed that their hatch could be influenced by *Alexandrium minitum* and *Gymnodinium,*with a significant dose-effect relationship was further found. In particularly, the mortality and teratogenicity rates which caused by *Gymnodinium minutum* were relatively higher than that by *Alexandrium minitum*. It was possible due to the penetration of certain substances through the egg membrane structure that generated by the algae. The results showed that the two high concentration alga solutions could inhibit the black sea bream larvae's weight and length growth more pronouncedly. The larvae's swimming activities and the normal metabolism could be weakened exposed to the algal cell sap, and thus the process of larval growth was inhibited. The presence of PSP neurotoxins in *Alexandrium minitum* might cause the nerve and muscle tissue disorder by blocking the sodium channels, and resulted in functional disease as well as the behaviour of abnormal activity [7].The normal physiological function might be affected for the depolarization of muscle and nerve cells, due to the fact that sodium ion flows could be induced by *Gymnodinium*[13]. With the extension of exposure time, the black sea bream could resist to such a poisonous effect in a certain degree, which was likely to be a physiological adaptive response.

Paralytic shellfish poison and *Gymnodinium* toxin ,as sodium channel receptor neurotoxins, might cause biological reactions retardation, spastic convulsions, respiratory paralysis, difficulty breathing and other symptoms[12].The larvae exposed to the high concentration algae fluids, in the 96 hours' acute exposure experiments, had shown distinct poisoning symptoms, such as reduced swimming ability, physical balance, tilt, and so on.

Na^+-K^+-ATPase was a special carrying enzyme required in the active transport, and was a major regulator of ion transmembrane transport, with its function to keep the balance of ions in the two sides of the membrane of tissues or organs [6].The ATP level would change for the external stresses, so the activities that supplied energy and ionic balance would be destroyed, and as a result, the ATP enzyme activity would change with it. In the experiment it indicated that the two algae fluids inhibited the activities of Na^+-K^+-ATPase enzyme in a dose-effect approach and in particularly, the toxic effect of *Gymnodinium* was more obvious. The response to a stimulate might have a certain relationship to the interdiction of the Na^+ and K^+ ionic flow balance, which resulted from toxins entering into fish bodies.

GSH-PX could deoxidize harmful lipid peroxides into hydroxyl compounds and decomposed peroxides so as to protect the cell membrane structure and function from being destroyed by peroxides [14]. All of the concentration groups did not show distinct dose-effect relationship in the inhibition to larvae's GSH-PX enzyme activity, except for the comparison between high concentration group and control group. However, the inhibitions from the two kinds of algae had a significant difference.

The difference between this two algae cell fluid's toxicity was significant, and the toxic effect on fish eggs and larvae from *Gymnodinium* was higher than that from *Alexandrium minitum.* Concerning their poisonous effects to the early developmental of black porgy, however, a large number of experimental studies were needed in the future to authenticate whether the BTX toxic substances produced by *Gymnodinium* cell sap was relatively stronger than PSP that generated by *Alexandrium*.

5. Conclusions

Based on our results, it could be concluded that the hatch of the spawns was sensitive to both kinds of the algae. And the toxicity results from *Alexandriurn minuturn* were inferior to that from *Gymnodiniurn*. The test of larvae 96-LC50 showed that larvae was more susceptible to the *Gymnodiniurn* and produced certain resistance to the toxicity of the algae. Both the *Alexandriurn minuturn* and *Gymnodiniurn* restrained body length and weight increase of the larvae. *Gymnodiniurn* exposure had a more significant effect on ATPase and GSH-PX enzyme activities of larvae. In conclusion, the effects on growth and development of *Sparus macrocephalus* spawns and larvae resulted from *Gymnodiniurn* was much higher than that of *Alexandriurn minuturn*.

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